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Biochemistry

γ-Fluoromethotrexate: Synthesis and biological activity of a potent inhibitor of dihydrofolate reductase with greatly diminished ability to form poly-γ-glutamates

(methotrexate/cytotoxicity/chemotherapy)

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ABSTRACT **A methotrexate (MTX) analog containing fluorine at the γ-carbon of the glutamate moiety, γ-fluoromethotrexate (FMTX), has been synthesized and evaluated for its biochemical and pharmacological properties. FMTX inhibition of dihydrofolate reductase from several sources is nearly equivalent to that shown by MTX. Most important, FMTX is an exceedingly poor substrate for folylpoly(γ-glutamate) synthetase, the enzyme that catalyzes the biosynthesis of the highly-retained, cytotoxic MTX polyglutamates. Uptake experiments in H35 hepatoma cells show that FMTX accumulates to approximately the same extent as MTX at steady state. The rapid efflux of both derivatives is also very similar. The major difference detected in cells between the two compounds is the meager glutamylation of FMTX, due to the electronegative properties of the fluorine adjacent to the potential amide-forming carboxyl group. Exposure of dividing cells to 50 μM MTX for 2 and 6 hr results in the formation of 55 and 130 nmol, respectively, of the polyglutamates (more than two glutamate residues)/g of cell protein. With FMTX these values were reduced by 98% and 93%, respectively. Growth inhibition studies show that MTX is only 12-fold more toxic than FMTX when the cells are exposed to each derivative continuously for 72 hr. When the exposure time is reduced, a greater disparity between the inhibitory effects is observed; with a 2-hr pulse, MTX is 2300-fold more effective than FMTX. These data correlate with the effects of pulses of FMTX and MTX on *de novo* thymidylate biosynthesis in intact cells. The results indicate that of the parameters examined, the vastly reduced toxicity of FMTX after its removal from the culture medium is best correlated with impaired glutamylation. The data strongly suggest that prolonged toxicity of MTX is a result of metabolic conversion to MTX polyglutamates and that these effects are far more dramatic in short-term than in long-term exposure to the antifolates.**

The first observation of the formation of γ-glutamyl derivatives of methotrexate (MTX: 4-amino-N^{10}-methylpteroylglutamic acid) in rat liver (1) suggested a new parameter in the pharmacology of antifolates. The crucial difference between the glutamylated derivatives and the parent drug was later shown to reside in the prolonged cellular retention of polyglutamates (2–10). Thus, when cells containing pools of MTX and MTX polyglutamates are placed in medium lacking MTX, they exhibit elevated levels of MTX polyglutamates for prolonged periods of time, during which unmetabolized MTX rapidly leaves the cell (10, 11). This property,

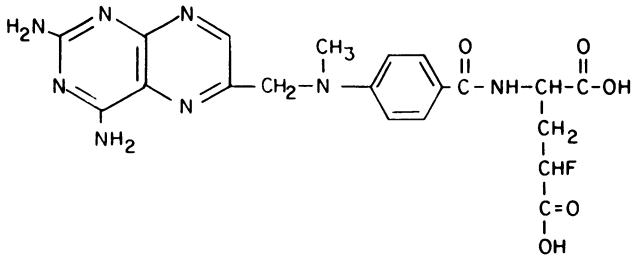

FIG. 1. The structure of FMTX.

coupled with an equivalent (5, 7, 8, 12) or possibly greater affinity (10) for the target enzyme dihydrofolate reductase (DHFR; EC 1.5.1.3), has led to the speculation that the MTX polyglutamates are the active agents in cells treated with MTX *in vitro* and *in vivo* (2–14).

Utilizing the H35 hepatoma cell culture system (3, 5, 6), we are attempting to definitively establish the role of glutamylation in the activity of MTX. The approach has been to employ a MTX derivative that is accumulated by cells and inhibits DHFR as effectively as does MTX but cannot be glutamylated. The derivative we have employed, γ-fluoromethotrexate (FMTX), is an analog of MTX containing fluorine in place of hydrogen on the γ carbon of glutamate (Fig. 1). Recent studies with partially purified folylpoly(γ-glutamate) synthetase (FPGS; EC 6.3.2.17) isolated from rat liver have shown that DL-*threo*-4-fluoroglutamate (*threo*-FGlu) acts as a chain-terminating inhibitor of the FPGS reaction (15). The data support the hypothesis that the incorporation of a molecule of FGlu into growing polyglutamate chains leads to a product, R-Glu-γ-FGlu, that undergoes further glutamylation at a markedly reduced rate. These results suggest that the synthesis of reduced folates or antifolates (e.g., MTX) containing FGlu in place of glutamate would lead to molecules with greatly diminished ability to form poly(γ-glutamate) derivatives. The topic of this report is the influence of the fluorine substitution on the toxicity of MTX in cultured hepatic cells. In particular, short-term exposure to FMTX causes growth inhibition that is several orders of magnitude weaker than that obtained with MTX. To establish that limited glutamylation is a major reason for these results, it is necessary to demonstrate (*i*) that both agents exert similar inhibitory effects toward the target enzyme DHFR, (*ii*) that FMTX and MTX are accumulated to a similar extent in H35 cells, and (*iii*) that FMTX is poorly glutamylated by H35 cells. These observations are evaluated with

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Abbreviations: MTX, methotrexate; FMTX, γ-fluoromethotrexate; FGlu, DL-4-fluoroglutamate; DHFR, dihydrofolate reductase; FPGS, folylpoly(γ-glutamate) synthetase.

regard to the potentiating effects of γ-glutamylation on the cytotoxicity of MTX.

MATERIALS AND METHODS

Materials. FGlu, supplied as a 1:1 mixture of the *threo* and *erythro* diastereomers, was purchased from Calbiochem. Swim's medium S-77 was obtained from GIBCO. MTX was generously provided by Lederle Laboratories (Pearl River, NY) and [3′,5′,7-^{3}H]MTX was obtained from Moravek Biochemicals (City of Industry, CA); both were purified prior to use by DEAE-cellulose chromatography (9) and the concentrations of their solutions were determined by UV spectroscopy (16). [3′,5′,7-^{3}H]MTX was adjusted to a final specific activity of 2–6 × 10^5 dpm/nmol. MTX-poly(glutamate) standards were kindly supplied by the National Cancer Institute. 4-Amino-10-methylpteroylglutamyl-γ-fluoroglutamate was prepared as described (15). *N*-Methyl-*p*-aminobenzoyl-γ-DL-4-fluoroglutamate di-*t*-butyl ester [N-CH_3*p*ABA-FGlu(O-Bu^t)$_2$] was synthesized from (*Z*)-*N*-CH_3*p*ABA (17) and FGlu(OBu^t)$_2$ as described for similar glutamate derivatives (18).

***N*-[4-([(2,4-Diamino-6-Pteridinyl)Methyl]Methylamino)-Benzoyl]-4-Fluoroglutamic Acid (FMTX).** A mixture of *N*-CH_3*p*ABA-FGlu(OBu^t)$_2$ (83 mg, 0.201 mmol) and 2,4-diaminopteridine-6-bromomethyl hydrobromide (18) (75 mg, 0.223 mmol) in Me_2NAc (2.5 ml) was stirred at 50–55°C for 4 hr and then left at ambient temperature overnight. Me_2NAc was removed under reduced pressure, giving 246 mg of crude product as a dark red-brown oil: λ_{max} (MeOH) 262 nm, 295 nm, 370 nm. This crude material was placed in an ice bath, and 2 ml of trifluoroacetic acid was added with stirring. After 10 min, the reaction mixture was allowed to warm to ambient temperature and was stirred for 4 hr. The trifluoroacetic acid was removed under reduced pressure giving the crude product (103 mg) as a light brown-yellow solid after trituration with Et_2O. The crude product was purified by chromatography on DEAE-cellulose with an NH_4HCO_3 gradient (15–600 mM). The material eluting at 300 mM NH_4HCO_3 accounted for the major UV absorbance and had spectral properties consistent with the structure of FMTX. Lyophilization of the column effluent containing the desired product gave a hygroscopic fluffy yellow solid (43.2 mg, 45% yield): λ_{max} (0.1 M H^+) 244 nm, 307 nm; (0.1 M OH^-) 257 nm, 302 nm, 370 nm. ^{1}H NMR (^2H_2O/^{2}HCl, pH 1) δ 2.30–2.94 (m, 2 H, β-CH_2), δ 3.30 (s, 3 H, NCH_3), δ 4.80 [d, 3 H, α-CH, CH_2-N(Me)], δ 5.42 (m, 1 H, γ-CHF), δ 7.08 (t, J = 10 Hz, 2 H, aryl), δ 7.73 (d, J = 7 Hz, 2 H, aryl), δ 8.63 (s, 1 H, aryl). ^{19}F NMR (^2H_2O, pH ≈ 6–7) δ −106.23 (multiplicity, ≈14). ^{19}F NMR (^2H_2O/^{2}HCl) showed two well-resolved septets, indicating an equal mixture of *erythro* and *threo* diastereomers. HPLC [Whatman SAX (19)] retention times were 30.2 min and 32.7 min (*erythro*/*threo* = 1:1).

Cell Culture and Drug Interaction Studies. H-11-EC3 cells (referred to as H35 cells) derived from the Reuber rat hepatoma were cultured as described (3, 9). The accumulation of [^{3}H]MTX was determined by the use of procedures previously described (3, 5, 11). The accumulation of FMTX was measured enzymatically with DHFR (20) by the procedures developed by Sirotnak and Donsbach (21). The conversion of MTX to MTX-poly(glutamate) derivatives was measured by using [^{3}H]MTX and HPLC (22). The possible metabolism of FMTX to polyglutamates was assessed by applying extracts of the cells to DEAE-cellulose columns (9). Although FMTX-poly(glutamate) standards are not currently available, the addition of γ-glutamate residues to FMTX would be expected to cause the derivatives to elute in the same position as the corresponding MTX-poly(glutamate). Thus, extracts of cells exposed to FMTX were evaluated for polyglutamates by pooling the combined regions of the chromatogram corresponding to MTX containing 3–6 glutamates, lyophilizing until NH_4HCO_3 was removed, and then assaying with DHFR (21). Control experiments using nonradioactive MTX showed that the DHFR procedure gave uptake, efflux, and metabolism data identical to those obtained with [^{3}H]MTX.

Growth-Inhibition Studies. The effects of MTX and FMTX on the growth of H35 cells were evaluated by using a Coulter Counter, as described (3), except with pulse doses of FMTX. Due to the high concentrations of drug required to effect growth inhibition, the short-term pulse experiments with FMTX were conducted on 2.0-cm^2 flat-bottom wells. Cell numbers were evaluated by counting the wells directly with a Zeiss invertoscope (23). This technique gives results comparable to electronic counting when MTX inhibition is compared under identical conditions (unpublished results).

RESULTS

Inhibition of DHFR. Comparable IC_{50} values are observed when the inhibitions by FMTX and MTX are compared for DHFRs from several sources (Table 1). However, FMTX is consistently a slightly poorer inhibitor than MTX of the H35 enzyme. Note that another fluorinated analog, the diglutamyl derivative of MTX with a γ-fluoro substituent on the terminal glutamate, has activity comparable to MTX. A greater difference was noted when the amount of MTX and FMTX needed to completely inhibit dihydrofolate reductase from H35 cells was measured. Approximately 5 times as much FMTX is required when compared to MTX, whereas 4-amino-N^{10}-methylpteroylglutamyl-γ-(4-fluoro)glutamic acid is equivalent to MTX (unpublished data).

Interaction with FPGS. FMTX was tested as a substrate for partially purified rat liver FPGS and was shown to have little activity when tested under standard assay conditions (Table 2). In addition, FMTX at concentrations many times that of MTX fails to substantially inhibit the FPGS-catalyzed formation of MTX polyglutamates.

Inhibition of Cell Growth by MTX and FMTX. H35 cells were exposed to MTX and FMTX either continuously throughout the culture period or for pulses of various times after the cells had been in culture for 24 hr (Table 3). The results indicate a somewhat reduced toxicity for FMTX (IC_{50} 12-fold that for MTX) when present throughout the culture period. However, the shorter the duration of the exposure, the greater the disparity in growth inhibition between MTX and FMTX. At the shortest time used (2 hr) MTX is 2300-fold more inhibitory than FMTX. Thus, the reduction in the exposure time to MTX from continuous (72 hr) to a short (2-hr) pulse causes only a 30-fold increase in the IC_{50} for MTX, whereas the same reduction in time increases the IC_{50} for FMTX by nearly 6000 fold. These results suggest that the

Table 1. Inhibition of DHFR by MTX and fluorinated derivatives

DHFR source*	IC_{50}, nM		
	MTX	FMTX	MTX-γFGlu†
H35 hepatoma cells‡	0.88	0.96	0.86
Human leukemic spleen§	0.75	0.95	0.75
MTX resistant-*Lactobacillus casei*¶	0.40	0.45	—

*Unless otherwise noted, the enzymes used are crude supernatants from lysates of cells grown as indicated in references cited below.
†4-amino-N^{10}-methylpteroylglutamyl-γ-(4-fluoro)glutamic acid (15).
‡Refs. 3 and 9. Assay (24) used 1.2–1.6 × 10^{-3} units at pH 7.5 and 37°C.
§Ref. 25. Assay (24) used 3 × 10^{-3} units at pH 7.0 and 37°C.
¶Ref. 21. Assay (24) used 1.0–1.2 × 10^{-3} units at pH 7.5 and 37°C. This partially purified enzyme was kindly provided by G. Maley (New York State Department of Health).

Table 2. Substrate and inhibitor activity of FMTX with FPGS

MTX, μM	FMTX, μM	Total glutamate incorporated, pmol	Relative activity,* %
100	—	5070, 4490	—
25	—	2320, 2330	100
—	100	190, 60	5
25	100†	2020, 2030	87
1‡	—	242, 230	100
1‡	20	211, 223	92
1‡	100	185, 186	79

FPGS was isolated from rat liver (26) and the partially purified enzyme was assayed as described (19, 26).

*Based on average values of the duplicates shown.

†FMTX concentration is 4 times that of MTX, since only the L-*threo* diastereomer of FMTX is presumed to act as a substrate (ref. 15; see text).

‡In a separate inhibition experiment, standard assays containing 1 μM [^{3}H]MTX (250 pmol; 3700 dpm/pmol) and the indicated concentration of FMTX were incubated for 4 hr. Products were analyzed by HPLC (19). The total glutamate incorporated was calculated from the amount of each polyglutamate species and the number of unlabeled glutamates ligated to [^{3}H]MTX to produce these species. Polyglutamates containing 1–3 additional glutamates were observed.

impaired capacity of FMTX to undergo the glutamylation reaction causes a reduced retention of this derivative after its removal, which in turn weakens its growth-inhibition properties.

Accumulation of FMTX and MTX by H35 Cells. In this study, we extended previous observations on the uptake and metabolism of MTX in H35 cells (3, 5, 6, 9, 11, 22) to include a comparison with FMTX. Confluent or dividing H35 cells were exposed to 5 μM MTX or FMTX for 2 hr (Fig. 2). For confluent cells (Fig. 2*A*), FMTX accumulation is nearly equivalent to that of unmetabolized MTX during steady-state accumulation (30–120 min), although the initial uptake of MTX is more rapid. The total MTX pool (MTX plus MTX polyglutamates) exceeds the cellular FMTX after a 2-hr incubation. The contribution of MTX polyglutamates became more apparent when the same experiment was conducted with dividing cells (Fig. 2*B*). Whereas MTX and FMTX accumulate to approximately the same extent, the relatively more rapid conversion of MTX to polyglutamates in dividing H35 cells (22) results in the total MTX pool exceeding that of FMTX by 5–6 fold.

Different rates of cellular release of MTX and FMTX could also alter the cytotoxicity of these derivatives. The efflux of these two analogs was measured for confluent H35 cells after a 30-min pulse of each derivative at concentrations of 5 μM (Fig. 2*C*) and 50 μM (Fig. 2*E*). In both cases slightly more FMTX than unmetabolized MTX is accumulated by 30 min. The losses of FMTX and unmetabolized MTX are similar in rate and extent. Slightly more MTX is lost, probably because it is displaced from DHFR by the MTX polyglutamate derivatives generated during the incubation (5, 7, 10, 12).

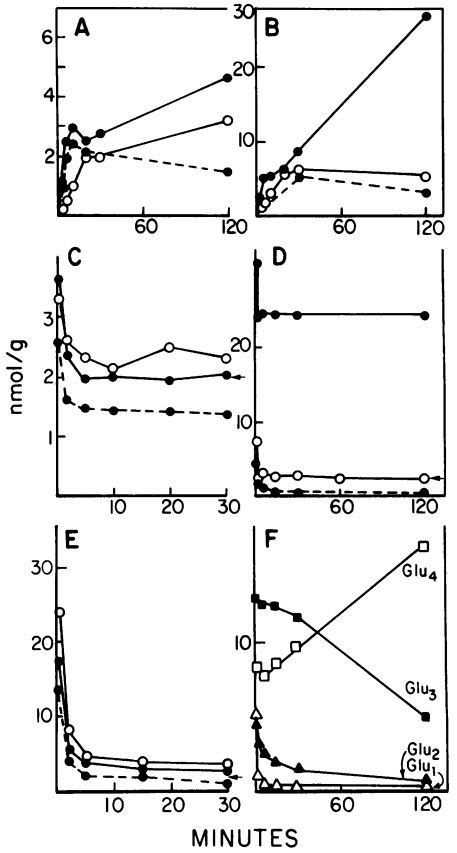

FIG. 2. Uptake and efflux of MTX and FMTX by H35 cells. The measurement of the cellular concentration of [^{3}H]MTX, [^{3}H]MTX polyglutamates, and FMTX are described in *Materials and Methods*. (*A* and *B*) The accumulation of 5 μM MTX or FMTX was measured in confluent (*A*, 144 hr in culture) and dividing (*B*, 48 hr in culture) H35 cells. FMTX measured by titration with DHFR is represented by open circles, MTX by the dashed line, and MTX plus MTX polyglutamates by the solid line. (*C* and *E*) The loss of the same components was measured after a 30-min pulse of 5 μM (*C*) or 50 μM MTX (*E*) to confluent cells. (*D*) An analogous study following a 2-hr pulse of 5 μM MTX and FMTX with dividing cells. (*F*) Kinetics for the individual MTX polyglutamates in the experiment represented in *D*. Subscripts indicate the total number of glutamate residues in the individual polyglutamate species. In *C–E*, the arrows indicate the amount of DHFR in H35 cells, which has been established previously (3).

Table 3. Effect of exposure time on the growth inhibition of H35 cells by MTX and FMTX

Exposure time, hr	IC_{50}, μM MTX	IC_{50}, μM FMTX	Fold difference
2	0.3	700	2300
4	0.1	34	340
7	0.06	10	167
24	0.02	0.4	20
72	0.01	0.12	12

H35 cells were cultured as described in *Materials and Methods* and the test compounds were present continuously throughout the culture (72 hr) or added after 24 hr in culture for the indicated time. Cell counts were done after 72 hr in culture. Data for 2 and 72 hr exposures are averages of four independent experiments with duplicate samples; the rest are means of two experiments.

The losses of FMTX, MTX, and the MTX polyglutamates was also measured after a 2-hr pulse to dividing cells. FMTX and MTX follow the same pattern of loss from the cell (Fig. 2*D*) observed in similar studies with confluent cells. Within 5 min, the cellular level of FMTX is reduced 70–75% to an amount just above the concentration of DHFR. The total MTX pool, which is much larger in dividing cells due to more rapid glutamylation, remains relatively stable for 2 hr after a slight reduction during the first 2–5 min. This is due to the predominance of longer polyglutamates (Fig. 2*F*) which are held within the cells for prolonged periods of time (11, 22).

Metabolism of FMTX. Studies with FPGS from rat liver have shown that folate or MTX analogs with fluorine in the γ-position of the terminal glutamate species are markedly compromised in their capacity to be glutamylated (ref. 15 and Table 2). To determine whether this is the case in intact

H35 cells, the conversion of MTX and FMTX to polyglutamates was compared. In these studies, glutamylation to form derivatives containing three or more glutamate residues was measured, since these are the major species and most avidly retained by the cells (Fig. 2*F*; refs. 8, 9, 10, 27).

A 2-hr pulse of 50 μM MTX to dividing cells results in the formation of 55 nmol of Glu_3 and higher homologs/g of cell protein, and this is increased to 130 nmol/g by 6 hr. The same amount of FMTX at 2 and 6 hr results in 98% and 93% reductions, respectively, in the formation of the same products, indicating the severe restriction of glutamylation caused by the γ-fluoro substituent. Quiescent H35 cells exhibited a much lower rate of glutamylation of FMTX, as is the case for MTX (Fig. 2 and ref. 22).

Effect of MTX and FMTX on *de novo* Thymidylate Biosynthesis. The relative rates of *de novo* thymidylate synthesis were measured by incubating H35 cells with [5-^{3}H]deoxyuridine after exposure to various regimens of MTX and FMTX (Table 4). As expected, both a continuous and a 2-hr pulse dose of 5 μM MTX, each of which are growth inhibitory (Table 3), cause a complete inhibition in ^{3}H release during the ensuing 24 hr. These results are consistent with previous studies on pulse exposures to MTX and the formation of polyglutamate derivatives (5, 11). Continuous exposure to the same concentration of FMTX also repressed *de novo* thymidylate formation.

We expected that concentrations of FMTX that are not toxic in a 2-hr pulse but do impair cell growth on continuous exposure should temporarily reduce ^{3}H release. Thymidylate synthesis should resume upon removal of FMTX from the medium, since few polyglutamates are formed and unmetabolized FMTX can rapidly leave the cell (Fig. 2). The lowest concentration used (5 μM) allows nearly complete resumption of ^{3}H release within 2 hr, whereas higher doses result in a more prolonged lag time before control rates are resumed. With 50 μM FMTX, this occurred between 2 and 4 hr after pulse, whereas with 300 μM FMTX, control rates did not ensue until after 6 hr of incubation in the drug-free medium. These results are consistent with the reduced toxicity of FMTX relative to MTX following pulse-exposure and confirm the relationship of growth inhibition (Table 3), *de novo* thymidylate biosynthesis (Table 4), and the intracellular generation of polyglutamates (Fig. 2).

Table 4. Inhibition of *de novo* thymidylate synthesis by MTX and FMTX

Treatment	Rate of ^{3}H release, cpm/0.1 ml of medium per hr			
	0–2 hr	2–4 hr	4–6 hr	6–24 hr
Control	219	194	250	294
Continuous				
MTX, 5 μM	0	0	0	0
FMTX, 5 μM	0	0	0	6
2-hr pulse				
MTX, 5 μM	0	0	0	0
FMTX, 5 μM	150	270	180	280
FMTX, 50 μM	5	218	335	277
FMTX, 300 μM	0	75	75	211

H35 cell cultures were grown for 48 hr, after which the *de novo* synthesis of thymidylate was assessed by incubating the cultures with [5-^{3}H]deoxyuridine and measuring the amount of ^{3}H released (5, 28, 29). For continuous exposure experiments, 20 μM [5-^{3}H]deoxyuridine (3.8×10^4 dpm/nmol) was added 2 hr after drug addition. In the pulse experiments, it was added after the MTX or FMTX was removed. The rate of tritium released was calculated by measuring the amount released during the indicated time period after addition of [^{3}H]deoxyuridine, and dividing by the number of hours in that period.

DISCUSSION

Recent investigations from a number of laboratories have indicated the widespread biological occurrence of the γ-glutamylation of MTX (1–14). Although the consequences of this metabolic conversion are not fully understood, a strong argument has been presented that implies that this process enhances growth-inhibitory effects of MTX (2–12, 14). This reasoning is based upon the observations that MTX polyglutamates are at least equivalent to MTX in the capacity to bind to DHFR *in vitro* (5, 7, 8, 10, 12) and are retained by numerous types of cells for much longer times than is MTX (2–11, 22). Thus, γ-glutamyl metabolites could exert antimetabolic effects after unmetabolized MTX has left the cell. However, there has not yet been a comparison of the growth-inhibitory properties of MTX with those of a nonglutamylated analog to determine the significance of glutamylation. To do this, the analog must have similar properties with regard to cell accumulation and DHFR inhibition. FMTX (Fig. 1) is a derivative that fits these qualifications.

MTX has an IC_{50} one-twelfth that of FMTX when H35 cells are exposed to both compounds on a continuous basis. The reason(s) for the difference is not established, but could be due to the stereochemical heterogeneity (DL-*erythro, threo*) of FMTX, modestly weaker activity of FMTX against DHFR (Table 1), and possibly the lack of glutamylation of FMTX. The difference in growth inhibition by MTX and FMTX is greatly amplified when the exposure is reduced to a 2-hr pulse (Table 3). This is presently best explained by the difference in the glutamylation of the two compounds. The reduced glutamylation is consistent with the observation that rat liver FPGS catalyzes a slow but measurable glutamylation of FGlu derivatives of MTX and tetrahydrofolate (15).

The data with FMTX underscore the importance of glutamylation in enhancing the cytotoxicity of MTX when cells or tissues are briefly exposed to the drug. In the absence of conversion of FMTX to the polyglutamates, a very limited inhibition of cell growth occurs. This is reflected in the relatively rapid reappearance of control rates of *de novo* thymidylate biosynthesis after pulse doses of FMTX, whereas thymidylate biosynthesis remains inhibited for 24 hr after a similar pulse exposure to MTX. Recent work by Goldman's group (30) with several cells with varying sensitivity to MTX, under conditions of continuous and pulse exposure to MTX, has led to conclusions similar to those presented herein.

It has been assumed that DHFR is the primary target of MTX, since MTX is a relatively poor inhibitor of other folate-dependent enzymes. The increased affinities of the polyglutamates for thymidylate synthase (31) and the transformylases of purine biosynthesis (32), however, suggest the possibility that these enzymes also may be involved in the cytotoxicity of MTX when polyglutamates are generated. The present study with FMTX cannot clarify this point but it does suggest that without glutamylation, insufficient amounts of unmetabolized MTX would be retained within the cell to inhibit these alternative sites.

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